Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 2785–2789, April 1996
Medical Sciences

Apoptosis of hemopoietic cells by the human granulocyte–macrophage colony-stimulating factor mutant E21R

(cell death/cytokines/progenitor cells/leukemia/receptors)

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Communicated by Seymour J. Klebanoff, University of Washington, Seattle, WA, December 11, 1995 (received for review October 30, 1995)

ABSTRACT **Human granulocyte–macrophage colony-stimulating factor (GM-CSF) binds to a high-affinity heterodimeric receptor composed of a specific α chain and a common β chain (β_c), which is shared with the receptors for interleukins 3 and 5. Hemopoietic cell survival requires GM-CSF binding this high-affinity receptor. We have recently developed the GM-CSF mutant E21R, which selectively binds to the α chain and behaves as a competitive GM-CSF antagonist. We have now examined the role of E21R on the survival of hemopoietic cells and found that E21R causes apoptosis (programmed cell death) of normal and malignant cells directly in the absence of GM-CSF. The direct apoptotic effect of E21R occurred in a dose- and time-dependent manner. Apoptosis by E21R was dependent on cells expressing the high-affinity GM-CSF receptor and could be blocked by GM-CSF. Significantly, apoptosis of the cells occurred even in the presence of the survival factors granulocyte CSF and stem cell factor but was prevented by engagement of β_c with interleukin 3. The initiation of apoptosis required phosphorylation, transcriptional activity, and protein synthesis. These findings support a model whereby binding of E21R to the α chain leads to apoptosis, while β_c plays an important role in cell survival. This model may be applicable to other multimeric cytokine receptors and offers a novel approach for the treatment of human leukemia.**

The human growth factor granulocyte–macrophage colony-stimulating factor (GM-CSF) exerts its biological action by binding to a high-affinity heterodimeric receptor complex composed of a GM-CSF specific α chain (GMRα), and a common β chain (β_c), which is shared with the receptors for interleukins (IL) 3 and 5 (1). Both chains are necessary and sufficient for GM-CSF-mediated signaling, as determined with cell lines transfected with the GMRα alone or with both GMRα and β_c (2). In keeping with the responsiveness to GM-CSF, human primary hemopoietic cells express the high-affinity GMRα plus β_c combination (3–5).

GM-CSF has been shown to contact both GMRα and β_c through two distinct faces in the molecule (6), and we have identified the residue Glu at position 21 in GM-CSF as crucial for the interaction of GM-CSF with β_c (6, 7). Substitution of this residue with Arg results in a GM-CSF mutant, E21R, which has retained full binding to the low-affinity GMRα but has lost its ability to bind to the high-affinity GMRα plus β_c complex (8). Moreover, E21R behaves as a specific GM-CSF antagonist as shown in binding experiments and functional assays (8).

One of the major functions of GM-CSF is to mediate survival (9) of normal and leukemic hemopoietic cells by suppressing apoptosis (programmed cell death) (10–12), and withdrawal of growth factors such as GM-CSF induces apoptosis of hemopoietic progenitor cells (10). Furthermore, several anti-leukemic compounds induce apoptosis of myeloid leukemic cells, and GM-CSF can rescue these cells from apoptosis (13). Previous data have mostly been obtained from experiments with defined cell lines (10–13), and little is known about the role of GM-CSF and its receptor subunits on the survival of human primary hemopoietic cells. We have now examined whether the GMRα specific mutant E21R could modulate survival of normal and malignant hemopoietic cells. In the present study, we show that E21R directly induces apoptosis of primary hemopoietic cells independent of the presence of GM-CSF and that an active mechanism is involved. The results indicate that the selective binding of E21R to GMRα directly promotes cell death and that engaging of β_c is important for cell survival. These results have implications for our understanding of cytokine receptor function and may offer a novel approach for treatment of myeloid leukemia.

MATERIALS AND METHODS

Preparation of Cells. Blood was collected from 21 patients with acute myeloid leukemia (AML; all Fab subtypes) (14) at diagnosis and the mononuclear cells were fractionated by density centrifugation (Lymphoprep, Nygaard). The T lymphocytes were removed by incubation with a mouse anti-human CD3 monoclonal antibody followed by a rabbit anti-mouse antibody coupled to magnetic beads (Miltenyi Biotech, Gladbach, Germany). This cell population consisted of >99% blasts. Bone marrow cells were collected from three healthy donors and fractionated; progenitors with the phenotypes $CD34^+CD38^+$ (lineage committed) and $CD34^+CD38^-$ (non-committed) (15) were sorted by flow cytometry ($FACStar^{PLUS}$, Becton Dickinson). We isolated the AML cells and the progenitors that expressed the GMRα using an anti-GMRα monoclonal antibody and the magnetic bead procedure. The fraction of GMRα-positive cells ranged from 50% to 75% in the 21 AML cases, and from 60% to 80% in the 3 $CD34^+CD38^+$ cases. These AML and progenitor cells also expressed the β_c. We could not detect GMRα among the three $CD34^+CD38^-$ cases (16). The cells were incubated in RPMI 1640 medium with fetal calf serum, antibiotics, sodium bicarbonate, and L-glutamine at 37°C. To the cultures we added either E21R (1 μg/ml; Bresatec, Adelaide, Australia) or GM-CSF (0.3 ng/ml; Genetics Institute, Cambridge, MA). In contrast to the AML cells that sustained at least 48-hr survival in medium alone (17), the bone marrow cells died within 24 hr if cultured in medium alone (10); thus, the latter were maintained with granulocyte colony-stimulating factor (G-CSF; 10 ng/ml; Amgen).

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Abbreviations: AML, acute myeloid leukemia; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte–macrophage colony-stimulating factor; GMRα, GM-CSF receptor α chain; β_c, common β chain; IL, interleukin; SCF, stem cell factor.

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Characterization of the AML Cells. By measuring the incorporation of [^{3}H]thymidine (8), we found that 12 of 21 AML cases showed GM-CSF-dependent proliferation. With the use of a sensitive (>10 pg/ml) ELISA (R&D Systems, Minneapolis) GM-CSF was detected in supernatants of only 8 of 21 AML cases.

Biological Assays. *Colony growth.* Colonies (>40 cells) of $CD34^+CD38^+$ and $CD34^+CD38^-$ progenitors in methylcellulose were prepared as described (18) and scored after 14 days. To triplicate cultures we added various combinations of E21R (1 μg/ml), G-CSF (10 ng/ml), GM-CSF (10 ng/ml), and stem cell factor (SCF; 10 ng/ml; Amgen).

Determination of apoptosis. The fragmentation of chromosomal DNA was displayed on an agarose gel after isolation of the DNA using an overnight incubation with lysis buffer followed by extraction with organic solutions as described elsewhere (11). The numbers of apoptotic cells were quantitated as reduced binding of propidium iodide to DNA using flow cytometry as outlined (19). Briefly, following incubation the cells were collected and kept in the dark at 4°C overnight in a buffer containing propidium iodide (50 mg/ml; Sigma) and Triton X-100 (0.1%; Sigma). Triplicate measurements were performed by flow cytometry.

Metabolic inhibitors. We used genistein (Sigma), a specific inhibitor of tyrosine phosphorylation (20), and the protein kinase C inhibitor staurosporine (Sigma) (21), which blocks phosphorylation of tyrosine, serine, and threonine residues. Transcription was inhibited with actinomycin D (Sigma), and protein synthesis was blocked with cycloheximide (Sigma).

RESULTS

E21R induces apoptosis in AML cells in the absence of GM-CSF. AML cells could be maintained in short-term culture conditions in medium alone. At the 48-hr time point, cells cultured in medium or GM-CSF showed no degradation of their DNA (Fig. 1). In contrast, in AML cells incubated with E21R alone for 48 hr, the DNA was degraded into low-molecular-weight fragments, a salient feature of apoptosis (22). To quantitate the proportion of cells undergoing apoptosis, we measured DNA binding of propidium iodide. We found that, in each of the 21 AML cases, at most 75% of unsorted AML cells underwent apoptosis with E21R. This proportion of apoptotic cells correlated well with the number of cells expressing GMRα. Upon purification of GMRα-positive cells with an anti-GMRα monoclonal antibody, virtually 100% of the cells were apoptotic after 48 hr when E21R was given at a dose of 1 μg/ml (Fig. 2*A*). This concentration was based on a titration experiment where 1 μg of E21R per ml yielded maximal apoptosis (Fig. 2*B*) and corresponds to a concentration that ensures full GMRα occupancy (8). The effect was dependent on E21R binding to GMRα, since a titration of GM-CSF against 1 μg of E21R per ml showed that 100 ng of GM-CSF per ml, a concentration that saturates the high-affinity GM-CSF receptor sites (8), totally abolished the apoptotic effect of E21R (Fig. 2*B*). GMRα-negative AML cells did not undergo apoptosis with E21R, indicating that E21R is not toxic (data not shown).

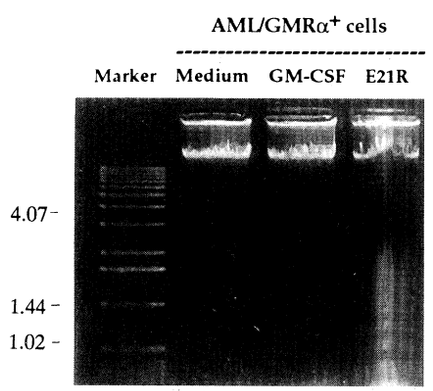

FIG. 1. The GM-CSF mutant E21R causes DNA fragmentation in GMRα-positive AML cells. Cells were incubated for 48 hr with either medium or GM-CSF (0.3 ng/ml) or E21R (1 μg/ml). Data are from one AML case and are representative of 20 other cases. Markers of known molecular sizes in kb are shown.

To examine whether the apoptotic effect of E21R was secondary to antagonism of GM-CSF present in the culture conditions or due to a direct effect of E21R, several controls were performed. First, it is known that while normal bone marrow hemopoietic cells do not express the GM-CSF mRNA (23, 24), some AML cells (23) can produce GM-CSF. However, GM-CSF was detected in supernatants of only 8 of 21 AML cases. The other 13 cases did not show detectable GM-CSF in the supernatants, and an RNase protection assay showed lack of mRNA for GM-CSF (data not shown). Second, biologically active GM-CSF in the culture medium was not present: enumeration of apoptotic cells in serum-free medium yielded similar results as depicted in Fig. 2*A*, and the neutralizing anti-GM-CSF monoclonal antibody 4D4 did not induce apoptosis of the AML cells (Fig. 2*A*). Finally, to determine whether any GM-CSF was bound to the AML cells when transferred *ex vivo* to the *in vitro* cultures, we measured the cell surface-associated binding of ^{125}I-labeled GM-CSF before and after acid elution. No differences were observed in specific GM-CSF binding, indicating that the cells were not carrying over receptor-bound GM-CSF.

Normal Myeloid Progenitors Undergo Apoptosis with E21R. To examine whether the E21R-induced apoptosis was a specific feature seen only in AML cells or dependent on GMRα expression, we studied the effect of E21R on survival of normal hemopoietic cells carrying the CD34 marker. Two subsets were studied: the $CD34^+CD38^+$ subset, which expresses GMRα, and the $CD34^+CD38^-$ subset, which does not express detectable levels of GMRα. Since initial experiments showed that deprivation of growth factors caused death among almost all progenitors within 24 hr, cell viability was maintained by adding G-CSF to the culture medium. Correlating with the GMRα expression, E21R caused apoptosis of the cells in the $CD34^+CD38^+$ subset but not in the $CD34^+CD38^-$ subset (Fig. 3*A*). DNA fragmentation was present in the $CD34^+CD38^+$ subset but not among the $CD34^+CD38^-$ cells (data not shown).

We next studied whether the apoptosis induced by E21R could be modulated by the presence of other growth factors known to influence cell survival. Importantly, apoptosis by E21R was seen even if two other survival factors, G-CSF and SCF, were present (Fig. 3*A*). However, when IL-3 was added to the medium, the $CD34^+CD38^+$ cells were rescued from apoptosis (Fig. 3*A*). Identical results were obtained with AML cells expressing functional receptors for G-CSF and SCF and grown with E21R plus G-CSF/SCF or IL-3 (data not shown).

The influence of E21R on growth of the progenitors was further determined in a colony assay. While G-CSF alone or combined with SCF supported colony growth, the addition of E21R markedly suppressed colony formation of $CD34^+CD38^+$ cells (Fig. 3*B*), consistent with E21R inducing apoptosis of these progenitor cells. Adding IL-3 to these cultures abolished the inhibition of colony growth induced by E21R (Fig. 3*B*). E21R had no effect on colony formation of the $CD34^+CD38^-$ subset (Fig. 3*B*).

E21R Induces Phosphorylation, Transcriptional Activation, and Protein Synthesis. Since E21R directly induced apoptosis, we examined the possible signaling requirements of this active process. A dose–response experiment showed that the specific tyrosine kinase inhibitor genistein did not affect E21R-induced apoptosis (Fig. 4*A*). In separate control experiments

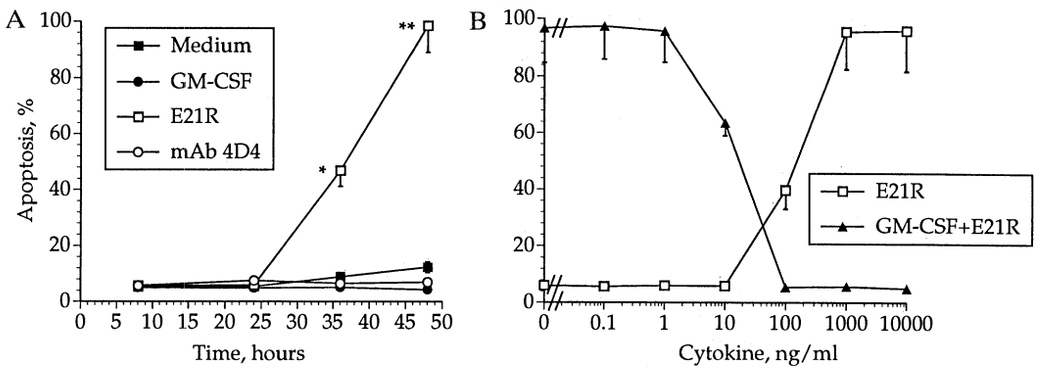

FIG. 2. E21R induces apoptosis of AML cells in a time- and dose-dependent manner. The proportion of apoptotic cells was determined by quantification of reduced DNA binding of propidium iodide by flow cytometry. (*A*) Time course of apoptosis in cells incubated with medium (■), GM-CSF (●; 0.3 ng/ml), E21R (□; 1 μg/ml), or the neutralizing anti-GM-CSF monoclonal antibody (mAb) 4D4 (○; 100 μg/ml). Mean values and SEM of data points from 21 AML cases are shown. Differences were evaluated by a two-way analysis of variance and Bonferroni's test. *, $P < 0.05$; **, $P < 0.01$ compared with medium. (*B*) Titration of E21R alone (□) and of GM-CSF (▲) in the presence of E21R (1 μg/ml). Values are means and SEM of three replicates from one AML case and are representative of 9 other cases.

genistein (0.1–10 μg/ml) blocked GM-CSF (0.3–1 ng/ml)-mediated stimulation of tyrosine phosphorylation in AML cells. The protein kinase C inhibitor staurosporine profoundly inhibited the E21R-induced apoptosis when given at a dose of 10 μg/ml (Fig. 4*A*), suggesting that serine/threonine kinases may be central to the apoptotic mechanism of E21R. Furthermore, we found that inhibition of transcription by actinomycin D or of protein synthesis by cycloheximide greatly reduced E21R-induced apoptosis of AML cells in a dose-dependent manner (Fig. 4*B*). Essentially the same results were observed with E21R-induced apoptosis of the normal $CD34^+CD38^+$ progenitor cells (data not shown).

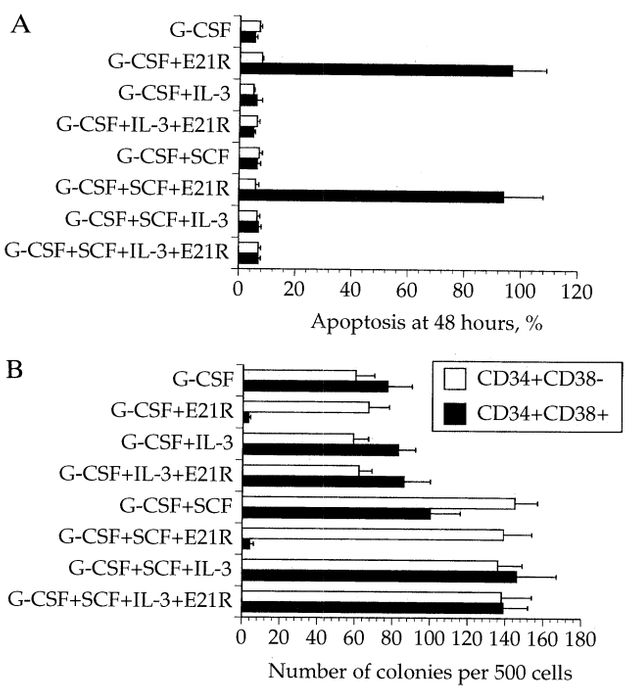

FIG. 3. E21R induces apoptosis of normal committed $CD34^+CD38^+$ (solid bars) but not of noncommitted $CD34^+CD38^-$ (open bars) hemopoietic progenitor cells. (*A*) Apoptosis at 48 hr of cells incubated with different combinations of G-CSF (10 ng/ml), SCF (10 ng/ml), IL-3 (10 ng/ml), and E21R (1 μg/ml). (*B*) Colony formation in the presence of different combinations of G-CSF, SCF, IL-3, and E21R used at concentrations shown in *A*. Data are means and SEM of triplicate samples from one case and are representative of two other cases.

DISCUSSION

We show here that the GMRα-selective mutant E21R directly induces apoptosis of both normal and malignant human hemopoietic cells. This is shown to be dependent on E21R binding to GMRα and the result of an active process that requires phosphorylation, transcriptional activation, and protein synthesis. Furthermore, E21R-induced apoptosis is shown

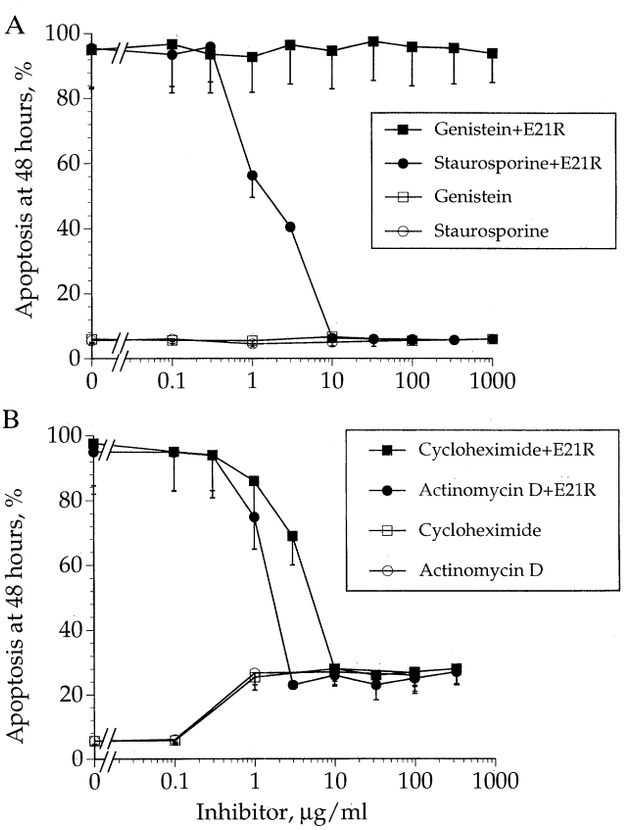

FIG. 4. E21R-induced apoptosis requires transcription, protein synthesis, and kinase activity. (*A*) Titration of genistein and staurosporine in the absence (open symbols) or presence (solid symbols) of E21R (1 μg/ml). (*B*) Titration of actinomycin D and cycloheximide in the absence (open symbols) and presence (solid symbols) of E21R (1 μg/ml). Apoptosis was measured at the 48 hr time point. Data are means and SEM of triplicate samples from one AML case and are representative of four other cases.

to be dominant over the survival effects of G-CSF and SCF but prevented by IL-3, suggesting an important role for the GM-CSF receptor in survival of hemopoietic cells.

The apoptosis induced by E21R was seen in AML cells as well as in normal $CD34^+CD38^+$ cells. Both normal $CD34^+CD38^+$ progenitor cells and AML cells stem from a small pool of cells with a high proliferative capacity (25–27) and appear to be equally sensitive to E21R. A key requisite for apoptosis was the expression of the high-affinity GM-CSF receptor indicating a receptor-specific event. This is consistent with the inhibition of E21R-induced apoptosis by GM-CSF. Furthermore, the absence of apoptosis in normal hemopoietic or AML cells lacking the GM-CSF receptor indicates that E21R is not toxic.

E21R induced apoptosis of hemopoietic cells in a direct manner independent of its receptor antagonist properties. Human GM-CSF protein and mRNA were absent in our culture conditions and a neutralizing anti-GM-CSF monoclonal antibody also failed to induce apoptosis. In addition, no surface-bound GM-CSF was observed in freshly explanted cells. Lastly, normal hemopoietic cells, which do not make GM-CSF (23, 24) and which had to be supplemented with G-CSF to maintain their viability, undergo apoptosis in the presence of E21R (Fig. 3*A*).

The exact molecular basis for the E21R-induced apoptosis is not yet known. Since E21R binds selectively to GMRα, it is possible that GMRα or the E21R–GMRα complex triggers a signal that leads to cell death. Alternatively, E21R may induce apoptosis by disrupting a preexisting and productive GMRα–β_c interaction. In support of the latter is the emerging evidence indicating that the GM-CSF receptor exists as a preformed complex (28), a finding consistent with the immediate high-affinity binding of GM-CSF in kinetic experiments (29). In contrast, the binding of IL-3 to its receptor is slow (29) and the IL-3 receptor α and β_c subunits are not intrinsically associated (unpublished observation). Since the basic residue His-367 in β_c appears to play a role in high-affinity binding, possibly by interacting with Glu-21 in GM-CSF (30, 31), the substitution of Glu-21 for Arg in E21R may lead to a charge repulsion that disrupts a preexisting GMRα–β_c interaction. Further support for this notion stems from experiments with receptor subunit-transfected Jurkat T cells showing that E21R induces apoptosis of GMRα plus β_c-transfected cells but not of GMRα only-transfected cells (data not shown). Thus, GMRα in isolation does not signal apoptosis. Notably, the Jurkat T cells grow autonomously in a factor-independent manner, and the E21R-induced apoptosis in these cells expressing the GMRα plus β_c receptor emphasizes the active nature of the apoptotic signal generated by E21R.

Regardless of the exact mechanism operating in E21R-induced apoptosis, it is clear that it involves an active process. Inhibitors of phosphorylation and of protein synthesis blocked apoptosis. Interestingly, while genistein, an inhibitor of tyrosine phosphorylation, was not effective, the addition of staurosporine inhibited apoptosis, implicating serine/threonine kinases in this pathway. A serine/threonine kinase has recently been found to be necessary for induction of apoptosis in Jurkat T cells (32), and it would be interesting to determine whether this molecule is also involved in E21R-induced apoptosis.

Since GM-CSF is not the only hemopoietic growth factor that supports cell survival, we examined the importance of GM-CSF and its receptor relative to other growth factors involved in hemopoietic cell survival—namely, the G-CSF, SCF, and IL-3 receptor systems. Importantly, E21R induced apoptosis in the presence of both G-CSF and SCF, both of which bind to homodimeric receptors distinct from the GM-CSF receptor (33, 34). This may be due to the fact that the E21R-induced apoptotic pathway is dominant over the survival signals generated by the G-CSF and SCF receptors. On the other hand, the survival effects of G-CSF and SCF may be exerted indirectly through the GM-CSF receptor. This could occur either by stimulation of GM-CSF production as shown in a myeloblastic cell line (35) or by phosphorylation of the GM-CSF receptor. This would be analogous to the stimulation of survival of erythroid precursors by SCF, which occurs by phosphorylation of the erythropoietin receptor by the SCF receptor c-kit (36). In contrast to G-CSF and SCF, IL-3 prevents apoptosis by E21R. This is likely to be the result of IL-3 engaging the β_c shared with the GM-CSF receptor. Taken together, these results point to β_c playing an important role in hemopoietic cell viability. This is consistent with the recent finding of suppression of apoptosis of hemopoetic cells by the wild-type human receptor β_c but not by its truncated forms (12).

Our data support a model whereby, although a preexisting GM-CSF receptor $\alpha\beta_c$ complex may allow short-term survival, ultimately the absence of GM-CSF and other growth factors leads to apoptosis. The presence of GM-CSF, IL-3, G-CSF, or SCF allows long-term survival and proliferation. The addition of the selective GMRα mutant E21R may disrupt the normal and productive interaction between GMRα and β_c and lead to apoptosis even in the presence of G-CSF/SCF, while engagement of the β_c with IL-3 prevents E21R-induced apoptosis. This survival versus apoptosis dichotomy depending on the receptor subunit(s) engaged may be applicable to other members of the cytokine receptor superfamily. Furthermore, since a majority of normal myeloid cells express the IL-3 receptor, while only 6 of 21 AML cases expressed the IL-3 receptor, our findings suggest that E21R might be beneficial and safe in the treatment of those myeloid leukemias lacking the IL-3 receptor. In these cases, endogeneous IL-3 or a small amount of exogeneous IL-3 may protect normal hemopoietic cells from E21R-induced apoptosis. Finally, the E21R-induced apoptosis of committed ($CD34^+CD38^+$) but not of noncommitted ($CD34^+CD38^-$) progenitor cells is likely to be useful for enrichment of primitive progenitor cells for stem cell transplantation and gene transfer purposes.

We thank D. Haylock for assistance with sorting of the bone marrow cells. E21R was a gift from Bresatec (Drs. S. Bastiras and C. Cheah), GM-CSF and IL-3 were gifts from the Genetics Institute, and G-CSF and SCF were gifts from Amgen. This study was supported by the National Health and Medical Research Council of Australia. P.O.I. holds a fellowship with The Norwegian Cancer Society.

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